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Pectin Methylesterase from Kiwi and Kaki Fruits: Purification, Characterization, and Role of pH in the Enzyme Regulation and Interaction with the Kiwi Proteinaceous Inhibitor

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Pectin methylesterase was purified from kiwi (*Actinidia chinensis*) and kaki fruit (*Diospyros kaki*). The pH values of the fruit homogenates were 3.5 and 6.2, respectively. The kiwi enzyme is localized in the cell wall and has a neutral-alkaline pI, whereas the kaki enzyme is localized in the soluble fraction and has a neutral-acidic pI. The molecular weights of the kiwi and kaki enzymes were 50 and 37 kDa, respectively. The two enzymes showed a similar salt and pH dependence of activity, and a different pH dependence of the inhibition by the kiwi proteinaceous inhibitor.

KEYWORDS: Pectin methylesterase; kiwi; kaki; salt and pH dependence of catalysis; protein−inhibitor interaction; surface plasmon resonance

INTRODUCTION

Pectin is a main component of the plant primary cell wall, into which it is secreted as highly methylesterified polygalacturonic acid (*1*). Subsequently, it is modified during different developmental stages of the plant such as cell extension and growth, organ abscission, and following plant pathogen attack. Pectin methylesterase (PME) removes the methyl ester groups present on the carboxyl moiety of galacturonic acid, releasing acidic pectin and methanol. By reducing the degree of methylesterification, pectin may then become susceptible to other endogenous pectinases, such as polygalacturonase. Because PME is involved in both stiffening and loosening of plant cell wall, it also plays a key role in the process of fruit ripening.

On the basis of their different catalytic mechanisms, PMEs were initially divided into two groups, able to perform "alkaline, linear demethylesterification" and "acidic, random demethylesterification", respectively (*1*). More recently, it was suggested that in different pH conditions the same enzyme might use different mechanisms (*2*). Moreover, the enzyme activity can be regulated by the proteinaceous inhibitor of PME (PMEI), isolated from kiwi fruit (*Actinidia chinensis*) (*3*, *4*).

PMEs are usually bound to the plant cell wall and can be solubilized by high ionic strength extraction. They are monomeric proteins, with sizes in the range 32−35 kDa, and most of them have neutral-alkaline isoelectric points. Only a few acidic PMEs have been isolated (*5*), probably because they are only weakly adsorbed onto the cell wall components, and soluble-protein extraction procedures have to be carried out to increase their recovery (*6*).

The regulation of PME activity is of remarkable interest in agriculture, since it has been reported that this enzyme can mediate the propagation of phytopathogens, and in food industry, where it is responsible for phase separation and cloud loss in fruit juice manufacturing. Here, we report a study carried out on PMEs from two fruits showing a different pH of their juice, kiwi (*A. chinensis*) characterized by an acidic value (3.5), and kaki (*Diospyros kaki*) with a higher pH value (6.2), with the aim at understanding the influence of different cellular environments on the properties of this enzyme. Kiwi enzyme was extracted from the cell wall fraction and purified by a modification of the procedure already reported (*7*). In fact, different conditions of extraction and ammonium sulfate fractionation, and a final purification step of affinity chromatography on a PMEI-Sepharose column instead of heparin-Sepharose, were used. Kaki PME was obtained from the soluble fraction. The two enzymes were purified to homogeneity and biochemically characterized, and their binding properties toward PMEI were investigated by means of surface plasmon resonance.

MATERIALS AND METHODS

Kiwi and kaki fruit were purchased from local markets. Trypsin was from Boehringer; bovine serum albumin, dithiothreitol, Tris, bis-Tris, and 4-vinylpyridine were from Sigma; MES was from ICN; citrus pectin

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(60% degree of methylation) was from Fluka; and DEAE-cellulose (type DE52) was from Whatman. SDS-PAGE reagents were from BioRad. All other reagents were of the highest commercially available quality.

Protein concentrations were determined by the BIO-RAD Protein Assay (Bio-Rad), using bovine serum albumin as standard. SDS−polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 12.5% gels. Isoelectrofocusing was carried out with a PhastGel apparatus (Pharmacia), in the pH range 3−9, according to the manufacturer's instructions.

Purification of PMEI was carried out as previously described (*3*). The PMEI-Sepharose support for affinity chromatography purification of PME was prepared by binding PMEI to a CNBr-activated Sepharose. Gel filtration was carried out with a FPLC apparatus either on a Superdex 75 HR 10/30 or on a Superose 6 HR 10/30 column (Amersham-Pharmacia), equilibrated in 50 mM Tris-HCl, pH 7.5, containing 0.25 M NaCl, recording the absorbance at 280 nm. When needed, protein samples were concentrated by ultrafiltration using Centriplus YM-10 filters (Amicon, Millipore).

Trypsin digestion of PME was carried out at an enzyme:PME ratio of 1:20 (w/w) in 0.1 M Tris-HCl, pH 8.5, at 37 °C for 2 h. Separation of peptides was accomplished by reverse-phase HPLC on a μBondapak-C_{18} column (Waters, 3.9 × 300 mm), using 0.1% trifluoroacetic acid (A) and 0.08% trifluoroacetic acid in acetonitrile (B) as eluents. Amino acid sequencing was performed with an Applied Biosystems Procise 492 automatic sequencer, equipped with on-line detection of phenylthiohydantoin amino acids. Partial unblocking of N-terminal residues was achieved by incubation in 30% trifluoroacetic acid at 55 °C for 2 h.

PME activity was routinely measured following the decrease of pH upon demethylesterification of pectin in a solution containing 0.1% citrus pectin in 0.1 M NaCl, adjusted at pH 7.5−8.0. In inhibition assays, PME reaction was started after 5 min of preincubation with PMEI. PME activity as a function of pH was determined on a MeterLab PHM 290 pH-stat equipped with a ABU 901 autoburet and a SAM 90 sample station (Radiometer, Copenhagen), at ambient temperature (approximately 20 °C), in the pH range 5.0−8.5, in 5 mL sample solutions containing 0.2 μg of enzyme, 0.1% citrus pectin, and 10 mM bis-Tris-HCl or Tris-HCl buffer (pH ranges 5.0−7.0 and 7.5−8.5, respectively), in the absence and presence of 100 mM NaCl. Titration of released protons was carried out with 10 mM NaOH. One activity unit is defined as the amount of enzyme releasing 1 μmol of protons per minute.

The interaction of kiwi and kaki PMEs with PMEI was studied in real time by SPR using a BIAcore X (BIAcore, Uppsala, Sweden), analyzing PMEI binding to PMEs immobilized on a sensor chip. We also tried the reversed geometry of immobilization, but attempts to perform kinetic studies on immobilized PMEI were unsatisfactory probably due to an unfavorable orientation of the inhibitor on the sensor chip. Experiments were carried out by recording the changes in resonance units (RU), which are proportional to the mass of protein binding. Random amine coupling of both PMEs was carried out by injecting proteins (4 μg/mL each) in 10 mM sodium acetate, pH 4.7, following preactivation of the carboxymethylated dextran matrix (CM5 sensor chip) using *N*-hydroxysuccinimide/*N*-ethyl-*N*′-[3-(diethylamino)-propyl]carbodiimide. BIAcore X sensorgrams were recorded at a flow rate of 30 μL/min at 25 °C, using sodium citrate pH 3.5, sodium acetate pH 5.0, Mes pH 6.0, Hepes pH 7.0, Tris-HCl pH 8.0, or CAPS pH 9.5 as running buffer. The analyses were performed using sensor chips with 800−1000 RU of immobilized protein. The sensorgrams were analyzed using BIAevaluation version 2.1 software, and k_{off} values were determined from the data collected during the dissociation phase ($dR/dt = -k_{off}R$).

PME activity assays and SPR measurements were carried out in triplicate, and an average standard deviation of ±3% for the obtained values was calculated.

RESULTS AND DISCUSSION

Purification of PME. Kiwi fruits were homogenized in water. The pH of the homogenate was 3.5. After centrifugation, the supernatant was discarded and the pellet, containing the cell wall fraction, was collected and homogenized again in the presence of 0.5 M NaCl, adjusted to pH 8.3. The PME activity was recovered in the salt extract of the cell wall, similar to most PMEs so far described (*1*, *7*). It was then precipitated by addition of ammonium sulfate (75% saturation). The pellet, redissolved and dialyzed against 10 mM Tris-HCl, pH 7.5, was loaded on a DE52 column equilibrated in the same buffer. The PME activity, eluted in the column flow-through, was then loaded on a PMEI-Sepharose column (1.5 × 10 cm) equilibrated in 10 mM Tris-HCl, pH 7.5. The column was washed with the equilibration buffer containing 0.5 M NaCl, and PME activity was eluted with 10 mM Tris-HCl, pH 8.5, containing 0.5 M NaCl.

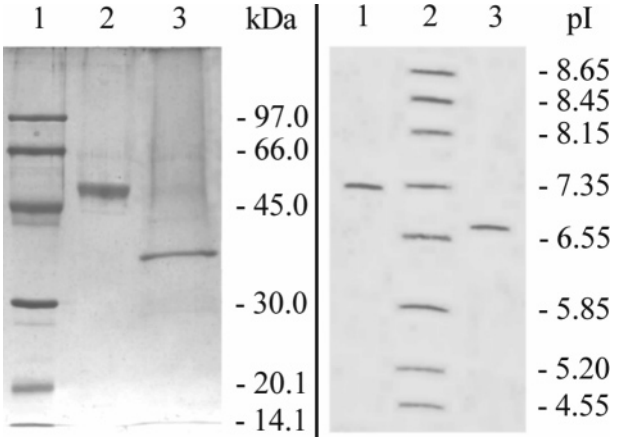

Figure 1. Left panel: 12.5% SDS-PAGE of kiwi (2) and kaki (3) PMEs; (1) low molecular weight standard proteins (Amersham Biosciences). Staining was carried out with Coomassie brilliant blue. Right panel: isoelectrofocusing of kiwi (1) and kaki (3) PMEs in the pH range 3–9, carried out as described in the Materials and Methods; (2) standard proteins.

Fully ripened, softened kaki fruits were homogenized in water. PME activity was detected in the supernatant (soluble fraction), which differently from kiwi and from most other fruits showed a less acidic pH (6.2). This fraction was precipitated with ammonium sulfate (80% saturation), and the pellet, redissolved and dialyzed against 10 mM MES, pH 6.0, was loaded on a DE52 column equilibrated in the same buffer. The PME activity, detected in the column flow-through, was loaded on a PMEI-Sepharose column (1.5 × 10 cm) equilibrated in 10 mM MES, pH 6.0. After loading, the column was washed with the equilibration buffer containing 0.5 M NaCl, and PME activity was eluted with 10 mM Tris-HCl, pH 8.5, containing 0.5 M NaCl.

Using these procedures, approximately 100−110 μg of electrophoretically pure PME (**Figure 1**) was obtained from 1 kg of kiwi or kaki fruits, with a purification factor of approximately 750-fold for both enzymes.

Isoelectrofocusing, Molecular Mass, and Amino Acid Sequencing of Protein Fragments. Similar to most plant PMEs, the kiwi enzyme has a neutral-alkaline pI (7.3, **Figure 1**, right panel), whereas the pI of the kaki enzyme is slightly acidic (6.7, **Figure 1**, right panel) and close to the pH of the pulp. This makes the kaki PME uncharged and unable to bind to the cell wall, and may explain why the kaki enzyme is released in the soluble fraction, probably during the ripening process.

The molecular weight of purified kiwi PME, determined by gel filtration on a Superose 6 column, was 50 kDa. A single band of molecular weight 50 kDa was detected by SDS-PAGE. The N-terminus of the polypeptide chain was blocked. The amino acid sequence of two internal tryptic peptides (QFYSECDVYGTIDFIFGNAAAVLQK and DDPNQNTGISILNCK) allowed a clear identification of the protein as a PME.

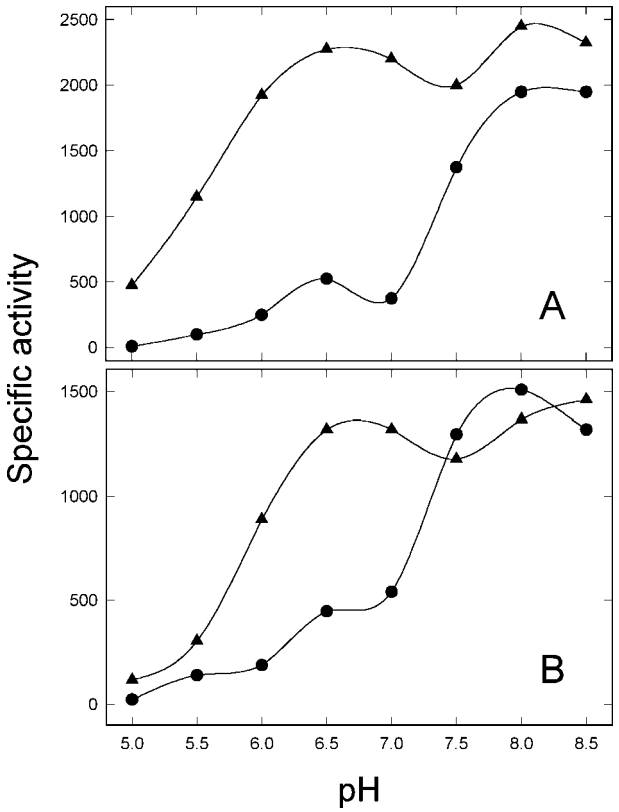

Figure 2. Specific activity (μmol/min/mg) as a function of pH of kiwi (**A**) and kaki (**B**) PMEs, in the absence (●) and presence (▲) of 100 mM NaCl. Experimental details are given in the Materials and Methods.

The molecular weight of native kaki fruit PME was 37 kDa, calculated both by gel filtration on Superdex 75 and by SDS-PAGE. The N-terminus that resulted was blocked upon Edman degradation. Partial unblocking obtained by acidic treatment suggested the presence of an acetyl-serine residue, similar to some other PMEs (*8*, *9*). The amino acid sequence of two internal tryptic peptides (DITFQNTAGPSK and NVVDGSTTFNSA) clearly identified this protein as a PME.

Both kiwi and kaki PMEs are monomeric, but, while the apparent molecular mass of the latter is similar to that of most plant PMEs (32−35 kDa), the former enzyme shows a larger size. A higher molecular size has been reported for PMEs, which have been suggested to be glycoproteins. The neutral PMEα isolated from mung bean hypocotyls (*Vigna radiata*) has a molecular size of 45 kDa (*2*); its partial primary structure (deduced from the nucleotide sequence) reveals the presence of four putative N-glycosylation sites, indicating that the enzyme may be glycosylated. Kiwi PME has already been reported to be glycosylated with a molecular mass of 57 kDa (*7*). The apparent molecular mass of the kiwi PME described in this paper is 50 kDa; therefore, based on the difference from the values reported above for most plant PMEs, glycosylation may in this case approximately account for 30% of the total molecular mass. This hypothesis is supported by additional data (not shown) on the primary structure of this enzyme that, although still largely incomplete, indicate the presence of N-glycosylated positions.

PME Activity. The curves of activity obtained for kiwi PME showed two optima at pH 6.5 and 8.0−8.5, both in the presence and in the absence of 100 mM NaCl (**Figure 2A**). At pH 8.0, a specific activity of 2450 and 1950 units/mg was calculated in the presence and absence of NaCl, respectively. At pH 6.5, the activity in the absence of NaCl was approximately 4 times lower than that measured in the presence of salt. At pH 5.0, in the presence of 100 mM NaCl, it was 5 times lower than that observed at pH 8.0−8.5, whereas in the absence of NaCl it was 200 times lower than that measured at alkaline pH.

Similarly, the activity of kaki PME showed two optima at pH 6.5−7.0 and 8.0, both in the presence and in the absence of salt (**Figure 2B**). The specific activity at pH 8.0, in the presence of 100 mM NaCl, was 1370 units/mg. In the absence of NaCl, the activity was slightly increased at the higher pH optimum, whereas it was approximately 3 times lower at the lower pH optimum.

The two enzymes share similar properties in the pH and salt dependence of their activity. The lowered activity at the lower pH optimum in the absence of salt may be due to the adverse effect of specific charged groups in the enzyme/substrate interaction. Three mechanisms of action have been postulated for PME: (i) single chain mechanism (SCM), where the binding of the enzyme to the substrate is followed by deesterification of all contiguous methyl groups on the homogalacturonan chain; (ii) multiple chain mechanism (MCM), where the enzyme−substrate complex dissociates after each demethylesterification, resulting in the conversion of a single residue for each attack producing randomly distributed free carboxyl groups; and (iii) multiple attack mechanism (MAM), where the enzyme catalyzes the conversion of a limited average number of residues for each attack (*10*). Plant PMEs are generally considered to work through SCM or MAM, producing differently sized clusters of free carboxyl groups on the homogalacturonan backbone. In the α and γ isoforms of PME from mung bean hypocotyls, a single chain mechanism (SCM) was postulated at pH 5.6, whereas a multiple attack mechanism (MAM) was required to explain the data at pH 7.6 (*11*). Similarly, a different action pattern at pH 4.5 and 7.0 was suggested for apple PME (*10*). Should this be the case also for kiwi and kaki PMEs, it could be hypothesized that SCM, occurring at acidic pH, would be salt-dependent, whereas MAM, occurring at pH values above the neutrality, would be salt-independent.

PMEI-Binding Experiments. Binding experiments, performed by SPR, showed that PMEI strongly interacts with immobilized kiwi PME (**Figure 3A**), as indicated by the extremely low dissociation rates observed at pH values ranging from 3.5 to 8.0. The k_{off} at pH 9.5 was 3.0×10^{-3}. PMEI was completely released with 50 mM CAPS pH 10.0. Attempts to dissociate the kiwi PME−PMEI complex at lower pH values, varying the ionic strength and using different regeneration buffers, were unsuccessful. On the contrary, PMEI was completely released from immobilized kaki PME with 50 mM phosphate pH 7.5. The stability of the complex was greatly affected by pH (**Figure 3B**). A K_D of 8.3 nM was measured at pH 6.0 with a k_{on} of 5.3×10^4 and a k_{off} of 4.4×10^{-4}. At pH 7.0, the affinity was markedly decreased, mainly due to a faster dissociation ($k_{off} = 1.3 \times 10^{-2}$).

SPR experiments indicated a different influence of pH on the interaction between PME and PMEI. In line with the results reported for tomato PME, where a high dissociation velocity (k_{off}) of 7.7×10^{-3} at pH 7.0 was observed (*12*), the interaction between PMEI and kaki enzyme at neutral pH values is very weak. In fact, complete dissociation of the complex kaki PME−PMEI occurs already at pH 7.5, and the dissociation kinetics displays a marked change in a narrow pH range; k_{off} measured at pH 6.0 is approximately 30-fold lower than that measured at pH 7.0. In contrast, the complex kiwi PME−PMEI shows almost no dissociation in the pH range 3.5−8.0, and only at pH 9.5 is a k_{off} value similar to that reported for tomato PME at pH 7.0 observed. Complete dissociation occurs at pH 10.0, under

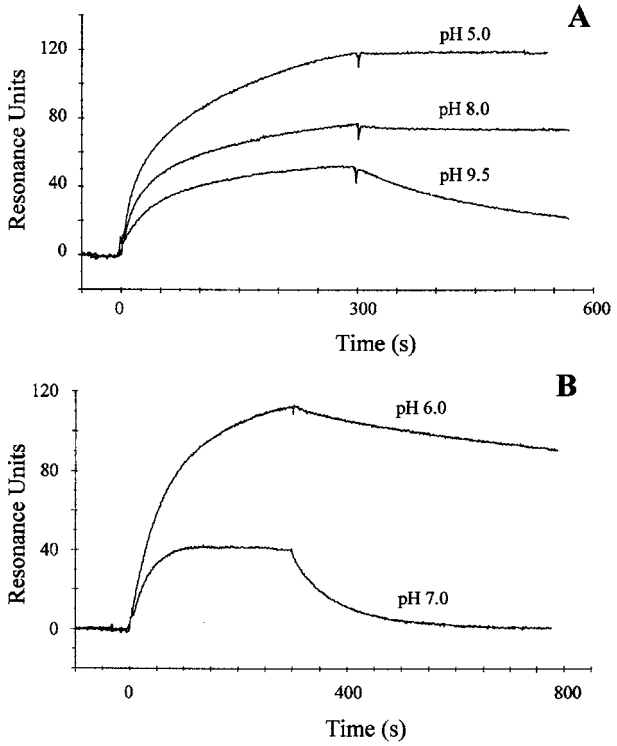

Figure 3. (**A**) Interaction between immobilized PME from kiwi fruit and PMEI at different pH values. PMEI concentration was 600 nM. (**B**) Interaction between immobilized PME from kaki fruit and PMEI from kiwi at different pH values. PMEI concentration was 300 nM.

conditions very far from the physiological ones. These data are in agreement with kiwi PME inhibition assays previously reported (*13*), showing that the inhibitor affects the enzymatic activity in the pH range 3.5−7.5.

It has been demonstrated that PMEI binds tomato PME at the active site (*14*). In this paper, we report a similar pH dependence of the catalytic activity and a different pH dependence of the protein−inhibitor interaction for kiwi and kaki PMEs. Therefore, it is conceivable that, although the two PMEs share some structural motifs in their protein−substrate and protein−inhibitor interaction sites, different structural features, probably related to the ionization state of specific groups, may affect the interaction with PMEI. The stronger interaction between PMEI and kiwi PME, that is its natural target, suggests the presence in the binding site of this enzyme of peculiar structural features, which are absent in homologous enzymes from other sources (kaki, tomato). The observation that only extreme pH conditions can dissociate the complex kiwi PME−PMEI may address the hypothesis that, in vivo, kiwi PME is irreversibly inactivated by PMEI when it has no further physiological function at the end of the ripening process. On the contrary, in conditions close to its physiological cellular environment, kaki PME interacts with PMEI very weakly. Therefore, while the catalytic activity appears similarly regulated in kiwi and kaki PMEs, the possible full inactivation of these enzymes at the end of the ripening process may occur only in the presence of different inhibitors or through alternative mechanisms, which may come into operation.

ABBREVIATIONS USED

CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; Mes, 2-morpholinoethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid; SPR, surface plasmon resonance.

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